Adenoviral-Mediated Herpes Simplex Virus-Thymidine Kinase Gene Transfer *in Vivo* for Treatment of Experimental Human Melanoma

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To assess the efficacy of an *in vivo* adenoviral-mediated cytotoxic gene therapy, human melanomas were established in nude mice and transduced with herpes simplex virus-thymidine kinase (tk) followed by treatment with ganciclovir (GCV). In initial experiments, adenovirus (adv) containing the β-galactosidase reporter gene was employed to determine melanoma cell infectivity *in vitro*. In comparison to murine melanoma cell lines B16 and K1735-M2, human A375-SM cells exhibited up to a 10-fold greater susceptibility to adenoviral transduction, similar to the degree of infectivity found for human epidermal HaCaT cells. In addition, human A375-SM melanoma cells exhibited a greater sensitivity *in vitro* to the cytotoxic effects of transduction with tk-adv and treatment with GCV, which was mediated by a strong bystander effect. *In vivo*, intratumoral injection of relatively large human melanomas (160 mm^3) with 1.2 × 10^9 pfu of tk-adv, followed by intraperitoneal GCV treatment (60 mg/kg twice daily) over 4 days, typically resulted in a 50% reduction in melanoma growth rate compared to mock or untreated controls. Moreover, histometrical analysis employing a rigorous computerized imaging system revealed that the residual viable tumor area in the tk-adv/GCV-treated group was only one-fifth that of solvent controls. These data show that adv is a highly efficient *in vivo* gene delivery system to treat experimental human melanomas. In comparison to a previous murine melanoma study, human melanomas appeared to exhibit a greater sensitivity to this cytotoxic treatment *in vivo*, which may hold significant promise for development of effective gene therapy modalities to treat melanoma in humans. *Key words: cancer/gene therapy/adenovirus/nude mice. J Invest Dermatol 106:1163–1168, 1996*

Melanoma is one of the most morbid of human cancers, because of its propensity for metastases and resistance to conventional treatment (Fidler, 1992; Johnson *et al*, 1995). While early localized disease can be cured by surgical excision, once the rapid vertical growth phase gives rise to distant metastases, surgery, chemotherapy, and radiotherapy have only a generally transient efficacy (Johnson *et al*, 1995). Indeed, for late disease (stage 4), no curative therapy exists. Given the alarming increase in the incidence of melanoma in caucasians over the past 50 years, predictions suggest that, in the United States alone, up to one in 75 individuals will develop cutaneous melanoma by the year 2000 (Rigel *et al*, 1987; Rigel, 1992). This fact, coupled with the prediction that ozone depletion is likely to increase the overall tumor incidence (Glass and Hoover, 1989; Gleason *et al*, 1993), makes the development of novel, effective treatment strategies highly desirable.

One potentially useful strategy is the application of somatic cell gene therapy techniques that transduce tumor cells with a variety of immunologic or drug-sensitizing "suicide" genes (Fujiwara *et al*, 1994; Hart and Vile, 1994; Vile and Russel, 1994). One example of the latter is transfer of the herpes simplex virus-thymidine kinase (HSV-tk) gene followed by administration of the drug ganciclovir (GCV). This normally nontoxic drug, an analog of guanosine, is phosphorylated by HSV-tk and converted to an intermediate that inhibits DNA synthesis by acting as a chain terminator (Matthews and Boehme, 1988) that kills dividing cells (Moolten, 1986). Thus, this approach is eminently suitable for the treatment of rapidly growing solid tumors that are invading normal tissues comprised largely of quiescent cells (Moolten, 1986). Furthermore, by employing this particular combination, not all tumor cells need to be transduced due to the existence of the "bystander effect" (Freeman *et al*, 1993), in which tumor cells in proximity to HSV-tk–transduced cells are also killed when phosphorylated GCV is released (Freeman *et al*, 1993).

To date, the majority of gene therapy treatment modalities rely on an *ex vivo* approach (Crowley and Seigler, 1993; Irie and DeNunzio, 1993; Foa *et al*, 1994; Porgador *et al*, 1994; Rosenthal *et al*, 1994; Rankin, 1995) in which autologous or allogeneic cells are

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Abbreviations: adv, recombinant, replication-deficient adenovirus; βgal, β-galactosidase; tk, herpes simplex virus thymidine kinase; βgal-, tk-adv, adenovirus containing the βgal or tk gene under control of Rous sarcoma virus long terminal repeat; GCV, ganciclovir; MOI, multiplicity(ies) of infection; pfu, plaque forming units.

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transduced by retroviral or nonviral means with cytokines or cell surface signal genes assumed to be pivotal in the elicitation or enhancement of an antitumor immune reaction. To be more useful in a clinical setting, however, direct delivery to tumor cells *in vivo* would be advantageous. Toward this goal, one attractive method involves the use of recombinant, replication-defective adenovirus (adv) (Chen *et al*, 1994), which can be produced at high titers, with the added safety feature of there being little significant potential for integration or insertional mutagenesis (Kozarsky and Wilson, 1993). Previously, adv-mediated gene delivery was used to successfully treat established murine melanomas with this suicide HSV-tk/GCV gene therapy approach (Bonnekoh *et al*, 1995). During the course of these experiments, we observed that murine B16 melanoma cells were relatively resistant to adv infectivity, which may have contributed to the continued tumor growth in treated groups (Bonnekoh *et al*, 1995). To assess whether this was a property of melanoma cells in general or restricted to murine cells and to determine whether this delivery system would be an effective treatment modality for experimental human melanomas, we now report that the human melanoma cell line A375-SM was 10-fold more susceptible to adv infection than murine cells *in vitro*. *In vivo* human melanoma growth rate was reduced by 50% after HSV-tk delivery and treatment of animals with GCV, and moreover, the residual viable tumor area was found to be significantly reduced to 20% of that of controls.

MATERIALS AND METHODS

Cell Lines The human A375-SM, the murine K1735-M2 and B16 melanoma cell lines were provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, The University of Texas, Houston) (Fidler, 1975; Kozlowski *et al*, 1984; Staroselsky *et al*, 1991), and the human HaCaT keratinocyte cell line was received from Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg) (Boukamp *et al*, 1988). The A375-SM, B16, and HaCaT cells were routinely cultured in Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum. The K1735-M2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and twofold vitamin solution (Gibco) (Staroselsky *et al*, 1991).

Recombinant, Replication-Defective Adenoviruses The construction and generation of adv serotype 5 containing the HSV-tk gene under the control of Rous sarcoma virus long terminal repeat has been described previously (Chen *et al*, 1994). Adv carrying the β-galactosidase gene (βgal) served as a control, the original stock of which was kindly provided by Dr. Michel Perricaudet (Institut Gustave Roussy, Villejuif Cedex, France) (Stratford-Perricaudet, 1992). Viral titers were determined by plaque assay on 293 cells and are expressed in plaque forming units (pfu) (Graham and Prevec, 1991).

Cell Culture Protocols In order to determine the cell line-specific sensitivity for adv transduction, 1.5×10^5 cells were plated in culture dishes with a diameter of 3.5 cm. Twenty-four hours later attached cells were trypsinized and counted in a hemocytometer. The subconfluent cultures were rinsed once with serum-free medium, and βgal-adv was added in a total volume of 1.5 ml of serum-free medium per dish at multiplicities of infection (MOI) ranging from 10 to 1000 pfu per attached cell. Three hours later 4 ml of fully supplemented growth medium (composition, see above) was added to each culture dish. At 24 h after onset of βgal-adv exposure, cells were fixed in 2% paraformaldehyde/0.2% glutaraldehyde (v/v) at 4°C for 10 min, and the number of cells transduced was assessed by staining cultures with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Fisher Biotech, Fair Lawn, NJ) and ferro/ferric isothiocyanate solution essentially as described (MacGregor and Caskey, 1989). The percentage of transduced blue cells was determined by counting 10 separate microscopic fields in triplicate.

The combined effect of tk-adv and GCV (Cytovene, Syntex, Palo Alto, CA) was studied *in vitro* by seeding 5×10^4 A375-SM cells per dish. After 24 h the cultures were incubated with tk-adv at a varying MOI under the conditions for βgal-adv described above. At 48 h after plating, the culture supernatant was replaced by 6 ml of growth medium containing 4 μg of GCV per ml or an appropriate volume of aqueous solvent (control). Five days after seeding, the number of attached viable cells was determined by trypan blue exclusion. This subtoxic concentration of 4 μg of GCV per ml treatment was determined by assessment of *in vitro* GCV toxicity alone in the concentration range 2 to 10 μg/ml.

***In Vivo* Experiments** Melanomas were generated in a subcutaneous site on the backs of 6– to 8–week-old athymic nude mice by injection of 2.5×10^6 A375-SM cells in 75 μl of phosphate-buffered saline under avertin anesthesia. At 2.5 weeks, when melanomas typically reached a volume of about 140 ± 20 mm^3 (mean ± SD) as determined by three-dimensional micrometer-caliper measurement, tk-adv, βgal-adv, or Tris-buffered saline alone were injected intratumorally. For this treatment animals were anesthesized with a ketamine/xylazine combination. Amounts of 6.0×10^8 or 1.2×10^9 pfu of adv dissolved in 40–50 μl of buffer were injected into the tumor center using a Hamilton syringe and a 30-gauge needle. To minimize leakage and to optimize the penetration of adv into the tumor tissue, injections were performed slowly (1 μl/15 s), and the needle was removed after a delay of 20 min. The next day, virus- and buffer-injected animals were randomized with respect to initial tumor size. During the following 4 or 6 d, GCV dissolved in isotonic sodium chloride was applied intraperitoneally at concentrations of 60 or 40 mg/kg body weight twice daily. Control animals received intraperitoneal mock injections with sodium chloride. Five or 7 d after the intratumoral adv injection the animals were sacrificed, the tumor volumes carefully measured, and tumor volume increase calculated with respect to the initial tumor volume at the day of adv injection.

Histochemic, Histopathologic, and Morphometric Analysis of Tumor Tissue To demonstrate βgal activity after adv-mediated gene transfer *in vivo*, tumor material was fixed by a 1-h incubation at 4°C with 2% paraformaldehyde/0.2% glutaraldehyde, incubated overnight with X-gal (MacGregor and Caskey, 1989), sectioned, and lightly counterstained with nuclear fast red. For histologic evaluation, tumors were fixed in Carnoy's solution (chloroform/acetic acid/ethanol, 3:1:6 v/v), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A computerized morphometric analysis with BIOQUANT software (BQ MEG IV; R&M Biometrics, Nashville) of the largest cross-sectional area of the residual tumor was performed by the point counting method because areas of necrotic tumor were not always contiguous. Briefly, at least 1600 predetermined points in the region of the tumor were assessed. From the total area of the tumor nodule (mm^2) and the relative amount of necrosis (%), the residual, viable tumor area (mm^2) was calculated for each tumor (Chen *et al*, 1995).

Statistical Analysis Statistical probabilities were obtained by the unpaired t-test and the Wilcoxon-Mann-Whitney U-test. The level of significance was set to $p < 0.05$, unless otherwise stated. Multiple comparisons required Bonferroni's adjustment (Bonnekoh *et al*, 1991).

RESULTS AND DISCUSSION

Transduction of Human and Murine Melanoma Cells by βgal-adv It is well known that there is a broad range of susceptibility of different host cell types for adv infection, with some tropism for the epithelial cells of the respiratory tract (Kozarsky and Wilson, 1993). Data concerning the susceptibility of melanoma cells for adenoviral infections are sparse, however, and relate mostly to murine studies (Zatloukal *et al*, 1994; Bonnekoh *et al*, 1995) that employed adv as recombinant vectors incorporating heterologous genes (Bonnekoh *et al*, 1995) or as carriers of noncovalently bound DNA complexes (Zatloukal *et al*, 1994).

In our previous report, we observed that B16 murine melanoma cells appeared to require a higher adenoviral titer to achieve 100% transduction of cells in βgal assays (Bonnekoh *et al*, 1995), than other tumor cells such as the C6 rat glioma cell line (Chen *et al*, 1994) or the MCA-26 mouse colon carcinoma cell line (Chen *et al*, 1995). To assess whether this was a general feature of melanoma cells (and therefore a potential problem), the infectivity of two murine (B16 and K1735-M2) and one human melanoma (A375-SM) cell lines was compared to a human nonmelanoma epidermal cell line, HaCaT. Cells were infected at a varying MOI with βgal-adv, and 24 h later the cells were fixed and stained with X-gal, and the number of βgal-expressing cells was determined. The results shown in **Fig 1** demonstrate that, in the range from 10 to 1000 pfu βgal-adv/cell, a concentration-dependent increase in cell transduction was observed for all four lines, with the highest transduction rates of 80% being found for the human HaCaT keratinocyte cell line and the human A375-SM melanoma cells (typical results are shown in **Fig 2*a***), at the maximum MOI of 1000

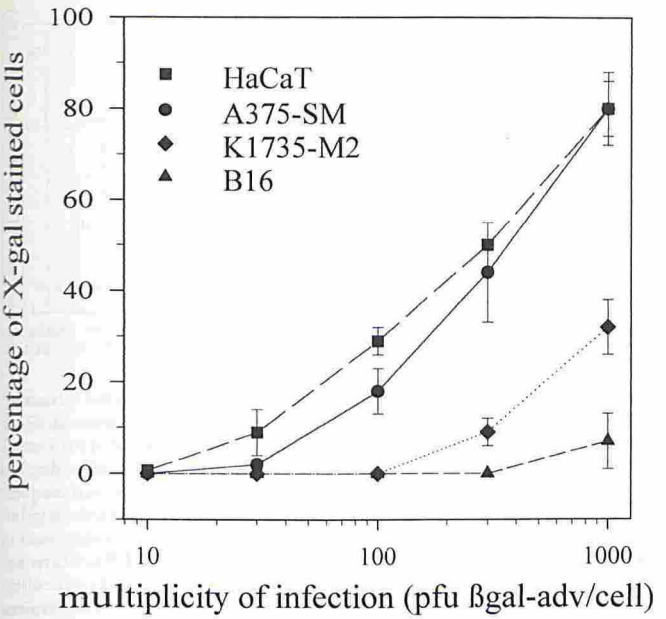

Figure 1. ***In vitro*** **transduction of cell lines by recombinant, replication-defective βgal-adv.** Human A375-SM melanoma cells, HaCaT keratinocytes, together with murine K1735-M2 or B16 melanoma cell lines, were incubated with βgal-adv at concentrations indicated. Twenty-four hours later the number of transduced cells was determined by X-gal staining (triplicate dishes, means ± SD). Significant differences for the adv transducibility of melanoma cell lines were observed, with the A375-SM being 2.5- to 10-fold more susceptible to infection with adv.

pfu/cell **(Fig 1)**. At this MOI, the corresponding average transduction rates for the murine K1735-M2 and B16 melanoma cell lines were 32% and 7%, representing a 2.5- and 11-fold lower infectivity, respectively, than for the human A375-SM melanoma cells.

Although the mechanisms underlying cellular adv transduction are certainly complex, the initial events of intracellular entry of adv are thought to be particularly important for the transducibility of a given cell type (Smythe *et al*, 1995). Basically, it has been shown that the carboxyl-terminal knob domain of the viral fiber protein initially attaches to a specific, as yet only partially characterized, cell surface receptor (Henry *et al*, 1994; Crystal, 1995). This event is known to be followed by a specific interaction between Arg-Gly-Asp motifs of the viral penton proteins and cellular vitronectin/fibronectin-binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, triggering the endosomal uptake of the virus particle (Greber *et al*, 1993; Wickham *et al*, 1993; Mathias *et al*, 1994; Huang *et al*, 1995). The latter integrin seems to also function in the subsequent release of the virus from the endosome into the cytoplasm (Wickham *et al*, 1994). In a recent study primarily focusing on the differential sensitivity of thoracic malignant tumor cells to adv transduction, a correlation with the cellular surface density of the α_v integrin could be excluded (Smythe *et al*, 1995). For melanoma cells, our finding of a broad range of divergent adv susceptibilities awaits future careful characterization of the cascade of critical cellular events from viral entry to nuclear translocation, transcription, and subsequent translation processes.

This increased infectivity of human versus murine melanoma cells was also observed *in vivo* and bodes well for use of adv to treat tumors in humans. Relatively large areas of intense X-gal staining were observed following intratumoral injection of established melanomas with βgal-adv **(Fig 2*b*)**. Injection speed proved to be critical (1 μl/15 s) for optimal transduction. In an attempt to assess the amount of adv required for transduction of tumors at a given size, based on the general assumption that a tumor mass of 1000 mm^3 contains 10^9 cells (Fidler, 1992) and the fact that the average sizes of the A375-SM melanomas in separate experiments were 165 and 122 mm^3 (see below), the tumors were injected with 1.2×10^9 and 6.0×10^8 pfu adv, respectively, corresponding approximately to an infectivity ratio of 7 and 5 pfu adv per tumor cell. Although these quantitative considerations give some clues to compare the effects observed *in vivo* and *in vitro*, consideration has to be given to the fact that *in vivo* free access of the adv to all of the target cells is not achievable, resulting in an uneven tissue distribution of tumor cell transduction as observed in the histologic evaluation of X-gal–stained sections of βgal-adv–treated melanomas **(Fig 2*b*)**. This observation may be somewhat offset, however, by the demonstration of a relatively strong bystander effect (see below).

In Vitro **GCV Cytotoxicity and Demonstration of a Bystander Effect in Human Melanoma Cells** Prior to assessment of the efficacy of adenoviral-mediated HSV-tk gene delivery and GCV cytotoxicity *in vitro*, the sensitivity of human A375-SM cells to GCV treatment alone was determined. Antiproliferative effects of GCV on eukaryotic cells are well documented in the literature, with an ID_{50} ranging from 10 to 65 μg/ml depending on the cell line and the assay conditions (Faulds and Heel, 1990). This influence of GCV on the proliferation of human A375-SM melanoma cells was tested by incubation with concentrations of GCV between 2 and 10 μg/ml for 3 d. Inhibition of A375-SM cell proliferation was observed with GCV concentrations ≥ 6 μg/ml (dose toxicity curve not shown); the average number of proliferating cells was reduced by ≥ 10%. From the dose toxicity curve, a concentration of 4 μg of GCV per ml was chosen as subtoxic and utilized for subsequent experiments. This result showed that human melanoma cells *in vitro* exhibited greater sensitivity to GCV than murine B16 cells, which exhibited no toxicity to GCV after incubation with 10 μg/ml for 6 d (Bonnekoh *et al*, 1995).

The cytotoxic potential of a combined tk-adv/GCV treatment on human A375-SM melanoma cells was first assessed *in vitro* by a co-incubation experiment. After transduction by tk-adv at MOI ranging from 0.1 to 3000 pfu/cell, the cultures were kept with or

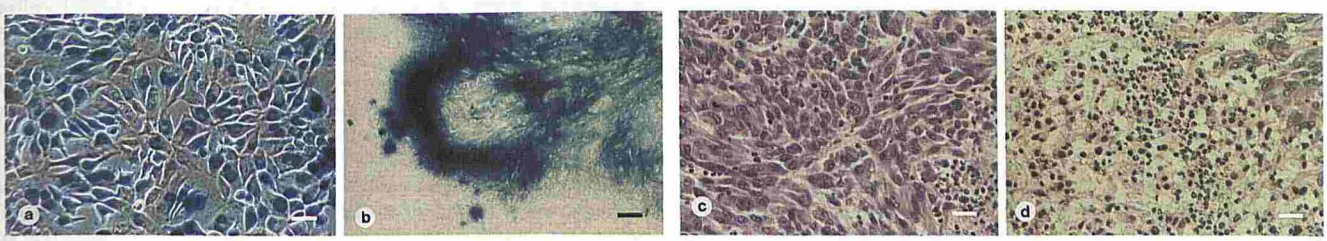

Figure 2. ***In vitro*** **and** ***in vivo*** **adv infectivity and histopathology of treated melanomas.** *a*, detection of βgal activity by X-gal staining in human A375-SM melanoma cells exposed to βgal-adv 24 h earlier at a MOI of 1000 pfu per cell. Note a majority of positive blue cells (*scale bar*, 3.5 μm). *b*, melanoma section stained with X-gal 2 d after intratumoral injection of βgal-adv demonstrates marked transduction of tumor tissue along the injection needle tract (*scale bar*, 60 μm). *c* and *d*, hematoxylin- and eosin-stained melanoma sections revealed that the βgal/ganciclovir (GCV) treated control melanoma possessed only minor necrosis and inflammation (*c*), whereas the HSV-tk/GCV-treated tumor exhibited extensive necrosis of the tumor tissue within 7 d (*d*) (*scale bar*, 30 μm).

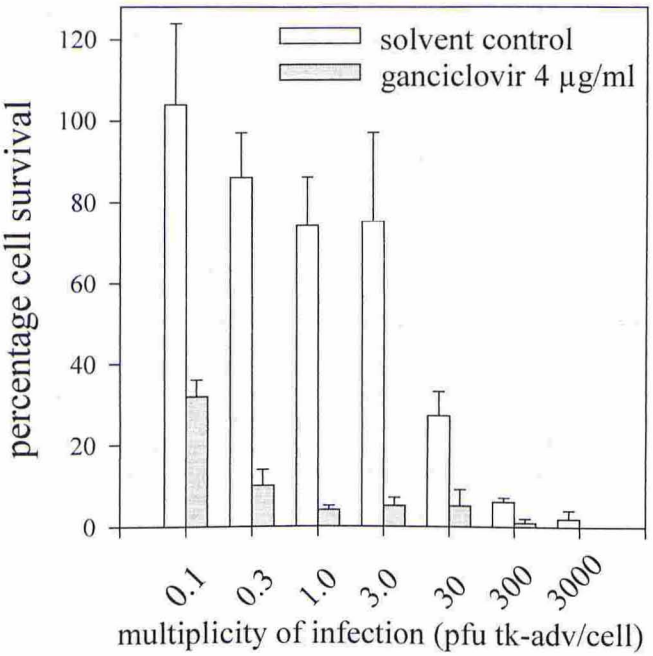

Figure 3. ***In vitro*** **transduction of human A375-SM melanoma cells with tk-adv and treatment with GCV.** Twenty-four hours after exposure to tk-adv, cells were cultured for 3 d with and without GCV at a concentration of 4 µg/ml. Cell survival was assessed as compared to adv- and GCV-free culture conditions, and significant cytotoxicity was observed at a MOI of 0.1 pfu tk-adv/cell in the presence of GCV. Note also a significant cytotoxicity of adenovirus at a MOI ≥ 30 pfu/cell.

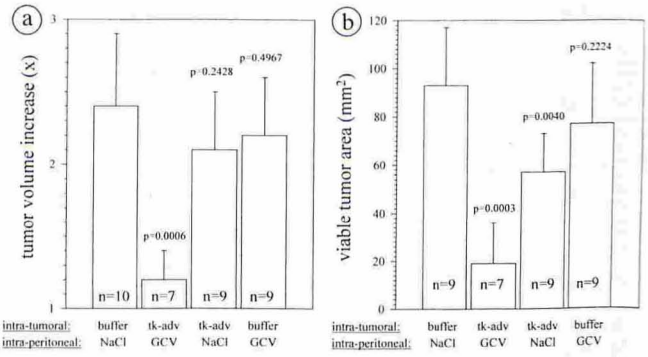

Figure 4. ***In vivo*** **inhibition of growth of established human A375-SM melanoma by intratumoral tk-adv/intraperitoneal GCV treatment.** Melanomas produced on the backs of nude mice (165 ± 57 mm^3) were slowly injected (1 µl/15 s) with 1.2×10^9 pfu of tk-adv, and GCV was given at 60 mg/kg body weight, twice daily, over the subsequent 4 d. The following day tumors were evaluated with respect to the individual tumor volume increase (*a*) and histometrically with respect to the area of viable tumor remaining (*b*). Results are expressed as means ± SD. Statistical analysis was performed by the Wilcoxon-Mann-Whitney U-test. Note that a significant (50%) growth reduction was observed in the tk/GCV-treated group, and also that an effect of adv infection alone was observed.

without GCV (4 µg/ml) for 3 d, then cell survival was determined. For the drug-free culture conditions, a significant cytotoxic effect of tk-adv infectivity was observed beginning at an MOI of 0.3 pfu/cell **(Fig 3)** and by an MOI of 30 pfu/cell the average cell survival dropped to 27%. Nevertheless, a clear effect of tk/GCV treatment was observed on A375-SM cells *in vitro*. At an MOI as low as 0.1, which exhibited no adv cytotoxicity, only 32% of cells survived; this percentage decreased to the background levels at an MOI of 1. Given an MOI of 0.1 pfu per cell, a maximum of 10% of cells would theoretically become infected, thereby clearly indicating the presence of the so-called bystander effect (Freeman *et al*, 1993).

To assess the strength of the bystander effect in an accompanying experiment, tk-adv–transduced and nontransduced A375-SM cells were mixed in a ratio of 5:95 and then cultured with 4 µg GCV per ml for 3 d. Under these conditions the survival rate dropped to 52% in comparison to nontransduced, GCV-treated control cultures. This strong bystander effect for adv-transduced human melanoma cell cultures is in agreement with other assessments of the relative strength of bystander effects, such as *in vitro* findings on murine K1735-C19 cerebral melanoma cells, where the retroviral transduction of one cell with the tk gene led to the destruction of ten neighboring cells (Wu *et al*, 1994).

In Vivo **Growth Inhibition of Established Human Melanomas** The encouraging increase in *in vitro* efficacy of the HSV-tk/GCV treatment on human melanoma cells with respect to B16 murine melanoma cells (Bonnekoh *et al*, 1995) was also borne out *in vivo*. Not only was HSV-tk/GCV treatment efficacious, but human melanomas also appeared to be relatively sensitive to virus infection alone. Two sets of experiments were performed on large groups of mice bearing tumors of differing average sizes.

In the first experiment, at 18 d after subcutaneous cell injection the melanomas typically reached a volume of 165 ± 57 mm^3 and were treated with 1.2×10^9 pfu tk-adv each, then evaluated after 4-d administration of GCV at a dosage of 60 mg/kg body weight twice daily. With regard to the parameters of tumor volume increase **(Fig 4*a*)** and residual viable tumor area (mm^2, **Fig 4*b***) this treatment regimen resulted in a highly significant effect in the group of animals treated with tk-adv/GCV compared to the solvent control group (application of buffer intratumorally and NaCl intraperitoneally). The tk-adv/GCV-treated group exhibited only a 1.2-fold increase in tumor volume compared to a 2.4-fold increase for the solvent control group. With respect to residual viable tumor area, a stringent morphometric-computerized histologic analysis (Chen *et al*, 1995) demonstrated that, not only had the tk/GCV treatment halved the tumor growth rate, but it also resulted in a reduction of viable tumor area to an average of 20% (19 mm^2) of the total tumor area compared to that of the solvent control (100%, 93 mm^2; **Fig 4*b***), i.e., only a fifth of the treated melanoma remained viable. Given the degree of heterogeneity observed on delivery of βgal-adv *in vivo* **(Fig 2*b*)**, this again indicates the existence of a strong bystander effect (typical histologic sections of treated tumors are shown in **Fig 2*c,d***).

In this experiment, involving a large number of animals, two other control treatment conditions were included: one group was treated with tk-adv intratumorally alone, without GCV treatment (NaCl intraperitoneally), and one group was mock-infected with GCV only. Interestingly, a significant effect (as compared to the solvent treatment control group) was observed for viable tumor area following tk-adv infection alone and intraperitoneal NaCl application. Here the histometric evaluation gave a viable tumor area of 57 mm^2 (61% of solvent control, $p = 0.0040$; **Fig 4*b***) and indicated an increased *in vivo* sensitivity of human A375-SM cells to the cytotoxic effects of adv infectivity, similar to that observed *in vitro* (above). Nevertheless, when a statistical analysis to directly assess the effects of basic adenoviral infectivity to that of tk/GCV treatment was performed by comparing the residual viable tumor area tk/NaCl data (100%) to that of tk/GCV treatment, a significant effect of the tk/GCV was again observed. Consistent with the presence of a strong bystander effect, only 33% of the tk/GCV-treated tumors remained viable in comparison with the tk/NaCl group ($p < 0.05$).

This effect of tk-adv infection alone prompted a careful analysis to contrast the effects of basic adenoviral cytotoxicity to that of tk/GCV treatment of melanoma. In this second experiment the tumors were also injected with fewer viral particles, 6.0×10^8 pfu of either tk-adv or βgal-adv, at 16 d after the subcutaneous tumor cell inoculation, when the melanomas reached a volume of 122 ±

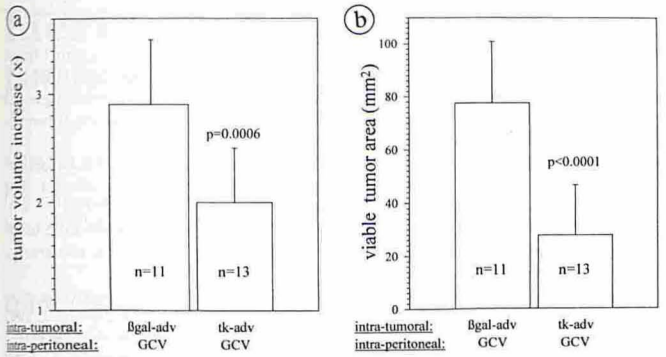

Figure 5. Comparison of the efficacy of tk/GCV treatment to infection with adv alone. The growth inhibitory effect of tk-adv/GCV treatment was compared directly to control βgal-adv infection and GCV treatment. The A375-SM tumors were grown to a volume of 122 ± 46 mm^3 and then injected with 6.0×10^8 pfu adv. After treatment with GCV (40 mg per kg body weight, twice daily for 6 d), the tumor volume increase (*a*) and the remaining viable tumor area (*b*) were determined. Data are presented as means ± SD with statistical analysis by the unpaired t-test. While an effect of adv alone was observed, the tk-adv/GCV treatment exhibited a significantly increased efficacy.

46 mm^3. Subsequently, the animals received less GCV, 40 mg per kg of body weight twice daily, and 7 d later the tumors were assessed. Under these optimized treatment conditions for both parameters, i.e., tumor volume increase and residual viable tumor area, a direct comparison of tk-adv/GCV treatment versus βgal-adv/GCV gave highly significant results ($p = 0.0006$ and $p < 0.0001$, respectively), as shown in **Fig 5*a,b***. The average residual viable tumor area was 28 mm^2 (36%) for the tk-adv/GCV condition as compared to 78 mm^2 (100%) with the βgal-adv/GCV control **(Fig 5*b*)**. Thus, a direct comparison with a rigorous βgal-adv control clearly demonstrated the therapeutic efficacy of the tk gene transfer, which produced gene function-related cytotoxicity significantly exceeding the adenoviral cytopathic effects. These data also show that a significant effect on both growth and residual tumor area can be achieved at lower GCV doses that begin to approach clinically acceptable levels (Faulds and Heel, 1990).

With respect to our observation of basic adv cytotoxicity in the absence of tk/GCV under the chosen conditions of locally high virus concentrations in the A375-SM melanomas, a previous report showed that transduction of cultured primary human airway epithelial cells with an E1,E3-deleted CMV-lacZ-adv vector resulted in increased apoptotic cell death and lowered recruitment of cells into S phase (Teramoto *et al*, 1995). It should be noted that these effects could be eliminated by ultraviolet inactivation of the vector, indicating that viral gene expression is involved in the slowing of cell growth.

Additionally, our histologic analysis revealed that the tk-adv/GCV-induced necrosis was always related to the injected tumor area. An injury of peritumoral normal tissue, e.g., dermal and subcutaneous connective tissue including blood vessels and the overlying epidermis, was not observed. An inflammatory infiltrate was virtually absent in untreated control tumors; however, some parts of the peripheral surviving tumor areas showed sparse infiltrates predominantly of neutrophils at day 7 after the onset of the treatment with tk- or βgal-adv/GCV, respectively. This finding reflects the athymic nature of the nude mouse model used in this study, while typically lymphocytic infiltrates have been reported after adv application in immune-competent animal models (Yang *et al*, 1995).

This study demonstrates that the growth of A375-SM melanoma in nude mice was significantly reduced by an adv-mediated intratumoral transfer of tk followed by GCV treatment, thereby providing evidence that human melanoma cells can be subjected to an efficient gene delivery *in situ* mediated by adv. Indeed, our data suggest that human A375-SM melanoma cells are more sensitive to the cytotoxic effects of tk-adv/GCV and the bystander effect than previously observed with murine B16 melanomas (Bonnekoh *et al*, 1995). This conclusion is based on both the observed increase in sensitivity of human melanoma cells to tk/GCV treatment *in vitro* and the observation that even given an effect of adv infectivity alone, the residual viable tumor area of treated human melanomas was reduced to one-fifth that of untreated controls. Thus, for treatment of melanoma, it may be that the efficacy observed in model systems with murine tumors underrepresents what may be achievable for humans.

Given this encouraging observation, our ongoing work,[1] which employs murine immune-competent, syngeneic melanoma models, has begun to explore the conditions under which the adv-mediated direct application of cytokines into a localized tumor compartment evokes optimum systemic anti-tumor protection (Rankin, 1995). This is clearly necessary, because a simple suicide gene approach cannot treat disseminated metastases; i.e., only local efficacy at the application site can be expected despite the actions of the bystander effect coupled to an increased sensitivity of human melanoma cells. As adv-mediated gene transfer has already been employed clinically for the treatment of cystic fibrosis (Crystal, 1995), successful immunotherapy or combination gene therapy identified in such animal model systems may be readily transferable to the clinical field of adjuvant melanoma treatment.

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